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Abstract

MicroRNAs serve a regulatory function in an array of cellular pathways. Using existing immunoprecipitation data that identified distinct differences in miRNA and gene enrichment in muscle cells in *C. elegans*, a preliminary RNAi screen was run to identify potential significant miRNA targets in muscle cells. The screen identified two potential genes, *twk-12* and *calm-1*, predicted to be regulated by miRNA-51. In order to determine the importance of miR-51 regulation of different muscle targets, RT-PCR was performed in a number of miRNA mutant strains and 3'-UTR reporters were constructed for *twk-12* and *calm-1*. Based on these experiments, *twk-12* may be regulated by mir-51, and *calm-1, twk-28*, and other important genes in muscle cells are likely subject to miRNA regulation. To determine any physiological consequences of miRNA regulation, ion assays were performed by subjecting worms to different concentrations of pharmacological agents that alter cellular ion concentrations. *Mir-51*(-) mutant worms proved to be more susceptible to altered calcium concentrations, suggesting a role of miR-51 in calcium regulation.

Introduction

MicroRNAs (miRNAs) are a family of single-stranded RNAs that are around 22 nucleotides in length and do not code for proteins (Lim et al, 2005; Bagga et al, 2005). Instead, they are involved in regulating gene expression in an array of pathways, including apoptosis, differentiation, neuronal activity, and tumor genesis (Ding and Han, 2007). Hundreds of miRNAs have been found in many different species of plants and animals, and more than 50% of the human genome is now thought to be targeted by miRNAs (Ding and Han, 2007).

After synthesis and modifications, miRNAs are associated into the miRNA induced

silencing comlex (miRISC). These complexes have been observed in a number of organisms, and consist of a number protein components, importantly the GW182 proteins AIN-1 or AIN-2 in *C. elegans* (Hafner et al, 2011, Tritschler et al, 2010). These complexes target messenger RNAs (mRNAs) that will eventually code for proteins. The target mRNA is recognized by a protein bound miRNA through complementary base pairing in the 3'untranslated region (3'UTR) of the mRNA. Recognition of target results in the prevention of translation into proteins or actual degradation of the mRNA targets by miRISCs (Bagga et al, 2005; Lim et al, 2005; Behm-Ansmant, 2006).

Since miRNA silencing mechanisms include a complex of various proteins, as well as a target mRNAs and miRNAs, an immunoprecipitation is a useful experimental method to study miRNA and targets. By pulling down AIN-1 or AIN-2 in *C. elegans*, the proteins in the miRISC can be isolated, as well as the miRNAs and mRNAs that are often associated with the complex (Zhang et al. 2007). Analysis of these miRNAs and mRNAs can indicate genes that are often targeted by miRNAs, as well as which miRNAs are commonly active regulators. This technique can be of further use to determine any differences in common mRNA targets in different tissues. By performing an IP of AIN-2 with different tissue specific promoters, former Postdoc Brian Kudlow was able to determine important differences in miRNA regulated genes in intestinal cells and muscle cells. Over 500 genes were specifically enriched in muscle cells, and ion receptors and channels proved to be enriched in muscle tissue (Kudlow et al., 2012).

In this project, pharmacological agents were used to assay responses to ion alteration. For these, agonists and antagonists of specific channels were used. Slo-like potassium channels are expressed in neurons, muscles, and other cell types. These channels are gated by voltage and calcium and are large conductance channels (Salkoff et al, 2005). Inhibition or stimulation of this

receptor would therefore alter intracellular potassium concentrations, potentially in a way to interfere with function. The ryanodine receptor is a calcium release channel in the sarcoplasmic reticulum that releases stored calcium during muscle contraction (Maryon EB et al, 1996). This results in a large influx of calcium to the cytoplasm when stimulated, and inhibition of this receptor would prevent this calcium release.

This project attempts to identify the role of miRNAs, specifically miR-51, in regulating ion transport and concentration in muscle cells. This hypothesis was derived from a study identifying tissue specific miRNAs and miRNA targets employing a co-immunoprecipitation approach by a previous postdoc in the lab, Brian Kudlow. This dataset identified many genes and miRNAs complexed with AIN-2, an important component in miRNA function, in muscle cells that are significantly enriched as compared to a full tissue AIN-2 IP (Kudlow et al 2012). This suggests the specific importance of certain miRNAs in muscle cells. From this data set, a number of genes were selected to perform an intial RNAi screen on *ain-1 (ku322); ain-2 (tm1863)* mutant worms. This strain is a complete loss of function of AIN-1 and partial loss of function of AIN-2; thus miRNA activity is significantly impaired. From this screen, a couple candidate genes were identified to be used in further study; *twk-12* and *calm-1*, both of which have predicted miR-51 binding sites. Based on this preliminary data, this project aims to determine the gene targets and physiological effects of miR-51 on ion regulation in muscle cells.

Materials and Methods

RNAi screen

An initial RNAi screen was performed using the common RNAi feeding method (Lamitina T, 2006). Potential miRNA targeted ion regulation genes were knocked down in an

ain-1(-);ain-2(-) double mutant strain. Since miRNAs always down-regulate, in strains with impaired miRNA function, it would be expected that expression levels of genes is higher than normal. If this high expression level is harmful, then knocking them down via RNAi could lead to a decrease in phenotypes. Genes were selected from a list of enriched genes related to calcium, potassium, and sodium regulation and function (Kudlow et al 2012). RNAi colonies were streaked from the ORF library and grown overnight on an LB plate with Ampicillin and Tetracyclin. From the stock plate, a single colony was inoculated into a 5 mL liquid culture and grown overnight in LB/amp/tet. This culture was spotted onto IPTG/amp plates and kept at room temperature for three days to dry and grow. On the fourth day, plates were either used or placed at 4˚C and used within two weeks. *C. elegans* with severely impaired miRNA activity ((*ain-1 (ku322); ain-2 (tm1863))* were placed on feeding RNAi plates and qualitatively assessed for overall health and mobility. From a preliminary screen, two candidate genes were identified: *twk-12* and *calm-1*. *Twk-12* is a potassium leak channel, and *calm-1* is a cytoplasmic calcium dependent protein that is thought to regulate synapse morphology (Salkoff et al, 2005; Caylor, R et al, 2011). The screen was replicated across three generations with phenotype scoring at the young adult stage; both uncoordinated (unc) and protruding vulva (pvl) phenotypes were recorded. The frequency of phenotypes was compared to the strain on a negative control, RNAi bacteria without a gene-knockdown vector. The data was normalized to this control.

3'-UTR Reporters

3'UTR reporters were constructed to assay miRNA activity of target genes *in vivo*. The 3'-UTR of the two candidate genes *calm-1* and *twk-12* were amplified via PCR from whole-worm lysate of wild type (N2) worms using primers with restriction endonuclease sites. Then, the PCR product was digested and ligated into the plasmid PPD129.57. This plasmid contained a gene

that codes for Green fluorescent protein (GFP), which can be observed through a fluorescent microscope filter. The 3'-UTRs of interest were inserted downstream of this gene. Since miRNAs act on the 3'UTR of target genes, effectively, this placed GFP expression under the control of miRNAs that regulate the native gene (Zhang, L et al., 2009). A red fluorescent protein (RFP) construct was included in the injection mix that was not subject to 3'UTR regulation. Thus, comparison of the RFP expression to GFP in the worm indicated whether or not a gene is regulated by miRNAs. A *rol-6* marker was also included in the injection mix to allow worms expressing the array to be identified. At this point, one line has been created expressing the *twk-12* 3'-UTR. It has already been crossed into a mir-51 loss of function mutant, (MT14450, n4473) and both strains have been scored. Microinjections to create more independent lines of *twk-12* and *calm-1* are ongoing.

RT-PCR

Both *twk-12* and *calm-1* were assayed via real time PCR using SYBR Green, in addition to a number of other genes with predicted miR-51 regulation. RT-PCR quantifies the level of a specific mRNA targets. Comparison in different miRNA knockdown strains can thus indicate any differences in gene expression related to miRNA function. Expression levels were determined in a number of miRNA mutant strains (mir-1(-), mir-51(-), mir-67(-), mir-231(-)) as well as wild type (N2) and *ain-1 (ku322); ain-2 (tm1863)* double mutants. The miRNA strains were selected based on predicted target sites as well as enrichment in muscle tissue. The genes being assayed were selected based on predicted regulation by mir-51 from the muscle IP data, and included *calm-1, twk-12, lin-17, twk-18, twk-28,* and *ZK822.5* (Kudlow et al 2012). Each strain was bleached to a synchronous culture, then RNA extracted at the L4 stage. Using Reverse transcriptase, cDNA was synthesized using an oligo dt primer to select only mRNA transcripts.

Primer sets were verified by standard curves with serial dilutions at each run, and a no template-control was run every replicate. The Ct counts for each were compared first to a loading control to correct any differences in initial cDNA concentration, *ama-1* or *rpl-26*, then to the wild type strain to determine any expression differences across strains. Data was not used if the primer standard curve did not have an r^2 of at least 0.99 or if the no template control CT count was 30 or below.

Pharmacological Ion Assays

In order to determine the physiological effects of ion mis-regulation, a number of assays were performed to alter the ion concentration within cells and see the effect of this alteration. In order to do this, four different pharmacological agents were purchased. The ryanodine receptor is a receptor that allows influx of calcium into the cytoplasm during muscle contraction. In order to alter intracellular calcium concentrations, worms were incubated with different concentrations of a ryanodine receptor agonist (4-chloro-3-methylphenol) and antagonist (dantrolene). Wild type or *mir-51(-)* worms were incubated for 30 minutes with different concentrations of these agents, then washed, plated and scored for uncoordinated phenotype (unc). Assays were run in three replicates with both wild type (N2) and mir-51 loss of function mutant. Agents to assay the effects of potassium are agonist (Rottlerin) and antagonist (Penitrem A) of the slo-1 K^+channel, a voltage-activated potassium channel (Crisford et al, 2011). Given the insolubility of these agents, aqueous solutions were made from stocks in dimethyl sulfoxide (DMSO), initially at 2.5% DMSO final concentration, then at 1.25% DMSO. A DMSO control was run alongside the agents to ensure that any observed phenotypes were separate from that caused by DMSO.

Results

RNAi Screen identifies genes that are likely miRNA targets

In the RNAi screen, there was a statistically significant reduction of phenotypes by the third generation. In the first and second generations, there is no statistical difference between gene knockdowns and the negative control (data not shown). However, by the third generation, many of the knockdowns do seem healthier than the control. There was a reduction in protruding vulva (pvl) phenotype in *calm-1, mec-2,* and *twk-18* ($p<0.05$); there was a reduction in the uncoordinated phenotype (unc) in *twk-18, twk-12,* and *calm-1* (Figure 1).

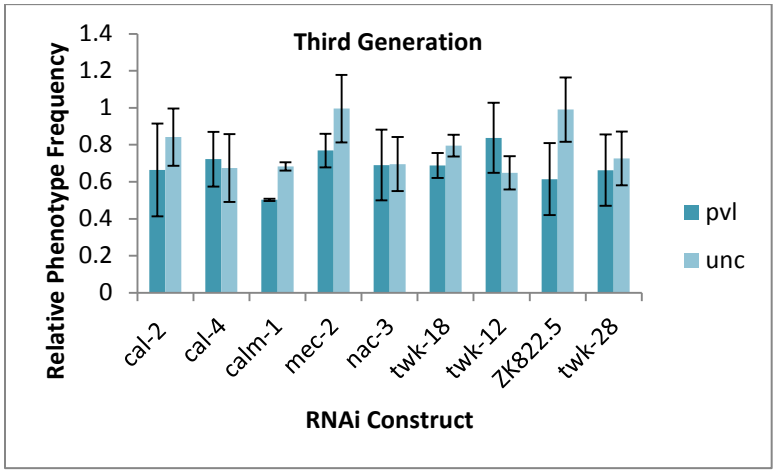


Figure 1: Worms with severely impaired miRNA function were placed for three generations on various RNAi constructs. Many of these constructs resulted in decreased frequencies of Unc and Pvl phenotypes compared to the negative control. (*p<0.05)

3'UTR-Reporter analysis detects potential regulation of twk-12 by miR-51

It has been shown that miRNAs silence gene expression by interacting with sites in the 3' untranslated region (3'UTR) of target genes. To provide key evidence that 3'UTR of *twk-12* and *calm-1* mediate the regulation by miRNAs, I carried out experiments to obtain transgenic animals that carries a *GFP::twk-12 3'UTR* or a *calm-1 3'UTR* reporter gene. I made both fusion constructs, but so far only one line had successfully been generated for GFP::*twk-12* (named *GFP::twk-12 3'UTR),* and none for *GFP::calm-1*. This single transgenic line has been scored. Looking specifically at body wall muscle cells, the expression of GFP was compared to RFP in the same cells. The expression was classified into three categories, comparable to RFP (=RFP),

less that RFP but still visible, and not visible to the naked eye (<<<RFP). In scoring the mir-51; *[GFP*::*twk-12 3'UTR*], it was observed that there were many worms that had a comparable brightness of GFP, but in fewer cells (about 50%). This was denoted as a separate category, and was not observed in any wild type; *[GFP*::*twk-12 3'UTR*] worms. Based on these observations, there is higher GFP expression in mir-51(-) worms than in wild type (Figure 2). It was also observed, although not quantitatively catalogued, that in a large majority of both worms, there was a similar amount of GFP in intestinal cells, but in the worm overall, GFP was much less expressed. This is consistent with a previous observation that *twk-12* is regulated by miRNAs using a 3'FUTR reporter in wild type worms (Zhang, L et al., 2009).

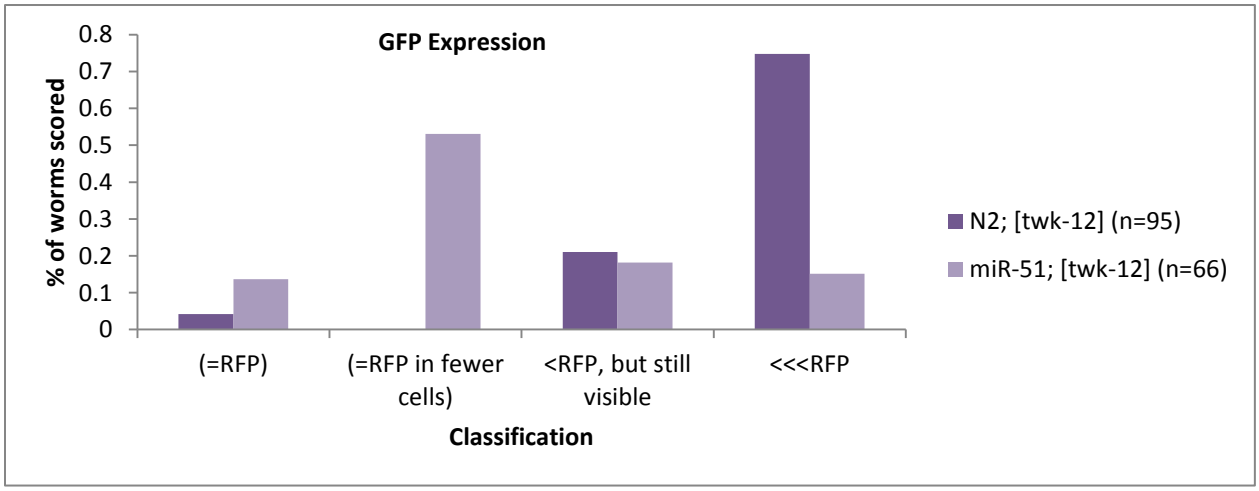
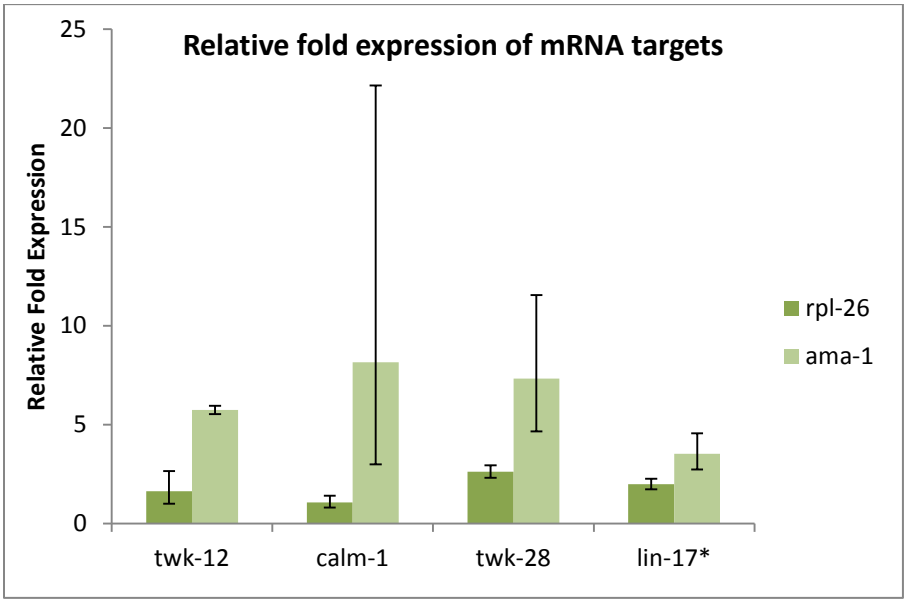


Figure 2: The GFP expression was observed in worms expressing the 3'UTR extra-chromosomal array. GFP brightness was compared to RFP to determine if there is miRNA regulation. Higher GFP expression was observed in mir-51(-) mutants than in wild type (N2), although still less than RFP.

RT-PCR indicates potential miRNA regulated genes

Since miRNAs act by preventing translation or degrading mRNAs of target genes, a quantification of mRNA transcripts can indicate possible miRNA regulation. For this reason, RT-PCR was performed to assay the level of mRNA transcripts of various genes in different strains. RT-PCR did not yield definitive results for all genes, rather the ddCT counts are quite

noisy for most genes (data not shown). However, when using *ama-1* as the loading control, *twk-28, twk-12, lin-17,* and *calm-1* all appeared to be higher expressed in the *ain-1(-); ain-2(-)* double mutant, consistent with miRNA regulation (Figure 3). This is repeated in the second loading control *rpl-26* for *lin-17* and *twk-28*. However, both *calm-1* and *twk-12* appear to have a difference in expression close to zero. In the individual miRNA mutants, there was no data to suggest which miRNA may regulate the genes due to the large discrepancies in values (data not shown).



*2 replicates

Figure 3: The relative fold enrichment of mRNA targets. This compares the expression level in the *ain-1(-); ain-2(-)* strain to the wild type strain. *Twk-12, calm-1, twk-28,* and *lin-17* all have a multiplied expression level in ain-1(-); ain-2(-) than in wild type.

Pharmacological ion assays indicate a calcium sensitivity in mir-51(-) worms.

In the calcium assays, it was observed that mir-51 mutants had a higher instance of uncoordinated worms at similar concentrations as wild type worms, with both the ryanodine receptor agonist and antagonist. The frequency of uncoordinated worms decreased with decreasing concentration of 4-chloro-3-methylphenol for both strains. For dantrolene, the trend

for mir-51 was not as defined, but there was still a clearer sensitivity to the agent of mir-51(-) worms (Figure 4).

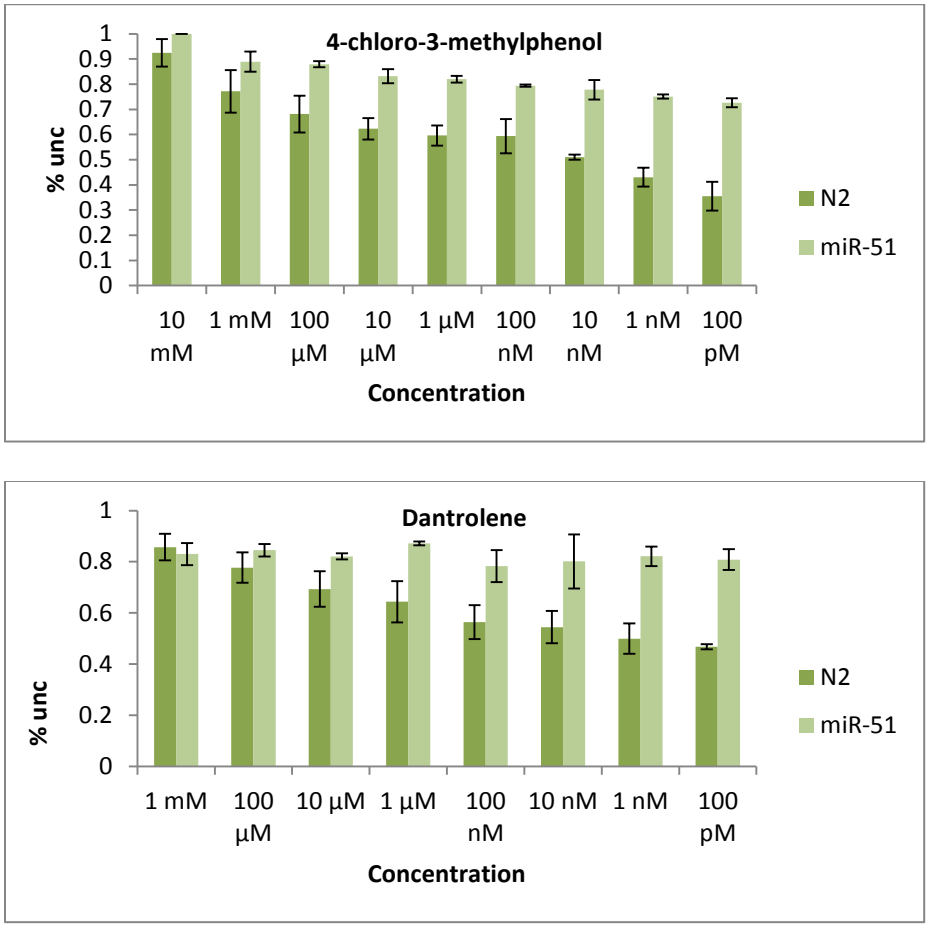


Figure 4: mir-51(-) mutant worms demonstrated a higher sensitivity to calcium-altering agents than wild type (N2) worms.

Standard wild type curves were generated for Penitrem A and Rottlerin as well, agents that alter potassium concentrations in cells. However, concentration of DMSO caused significant instances of uncoordinated worms in the control in mir-51 mutant worms (data not shown). The assays will be run using a lesser concentration of DMSO in the stock solutions.

Given the increased sensitivity of mir-51 mutants calcium altering agents, one rescue assay has been performed with mir-51 and wild type strains on one generation of *calm-1* RNAi.

There does appear to be a slight difference in the frequency of uncoordinated worms across the strains (Figure 5).

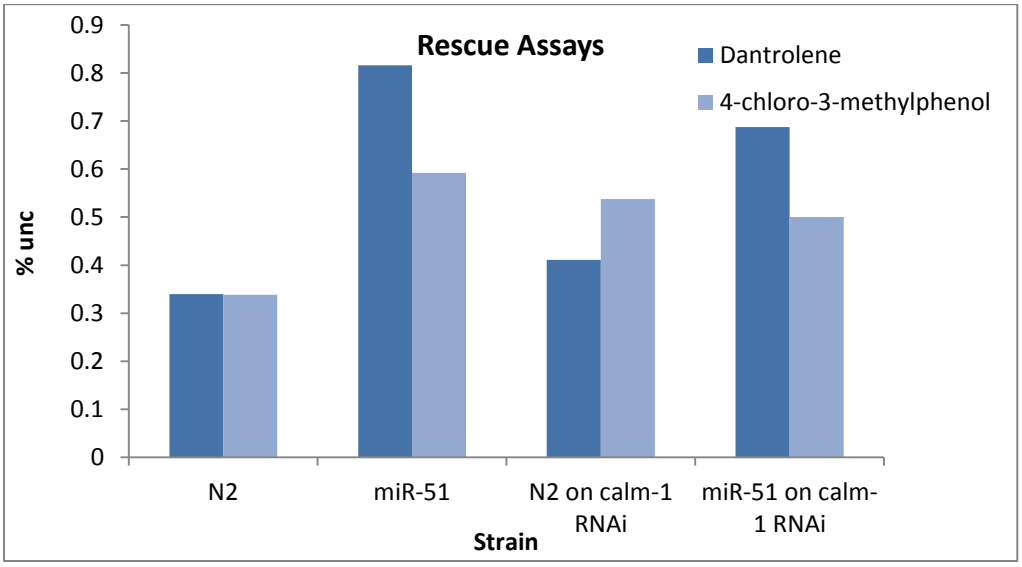


Figure 5: One rescue assay has been performed using RNAi to knockdown *calm-1*. There was a decrease in unc on the RNAi for the mir-51 strain.

Discussion

Based on the RNAi screen, it is likely that a few of the gene targets are regulated by miRNA or in pathways that are subject to miRNA regulation. Since the gene knockdown did reduce the instance of phenotypes, the mis-regulation of these genes or pathways does seem to cause harmful effects to the worm. While none of the differences were particularly large, this is to be expected given the large number of genes that are predicted to be regulated by miRNAs. It would not be expected that knocking down a single gene could drastically improve the health of this strain. The double mutant strain used in this screen is very unhealthy and difficult to work with. It is possible more statistically significant data could result from a substantial increase in sample size. Frequently, the plated worms to lay the next generation died before laying progeny.

Larger sample sizes would simply require picking many more worms per generation to avoid die off.

The RT-PCR data is not particularly conclusive, although it does suggest that a few genes are under the control of miRNA, like *lin-17, twk-28,* and maybe *calm-1* and *twk-12*. Much of the data is too noisy to be conclusive, and in the case of *calm-1* and *twk-12*, results fail to be conclusive for both loading controls. Furthermore, the results simply show that these genes are possible under the control of miRNAs, but since individual strains gave no conclusive data, there is no evidence as to which miRNA may serve as a regulator. A potential issue with the RT-PCR is that this project attempts to look specifically in muscle cell. Since RT-PCR is performed from total mRNA, it is not clear that any of this possible regulation is important in muscle. Furthermore, there could be important expression changes in muscle that are not displayed because there is lesser expression in other tissues. Overall, RT-PCR indicated potential genes of study, but did not give any real notion as to which miRNAs might be at play.

The 3'UTR reporter likely offered the clearest indication of miRNA regulation. Given the general higher expression in mir-51 mutants, it would seem that miR-51 does regulate *twk-12*. However, this result is not conclusive until the same pattern is observed in more than one line. Ongoing injections are important to confirm this result. The fact that many of the observed worms had comparable GFP expression in fewer cells suggests that the extra-chromosomal array may only be expressed in certain cells. It is important to generate more lines to be sure that this line is reliable. Furthermore, since no lines have been successfully made for *calm-1,* there is still no indication that miRNAs do regulate *calm-1* itself. For future studies, it may be useful to construct reporters for *twk-28* and *lin-17* as well.

The pharmacological ion assays offered an opportunity to not just probe any regulation of genes, but investigate the physiological consequences of this regulation. Given the higher sensitivity of *mir-51(-)* mutants to calcium altering agents, it seems that miR-51 does play some role in cellular calcium regulation. However, it is not clear at this point what genes play a role in this. The next step is to perform assays to that knock down genes of interest to see if this effect can be rescued to any extent. One such assay has been performed using *calm-1* RNAi, but more replicates are needed before any conclusions can be drawn. Furthermore, the assay was performed on worms that had been on RNAi for one generation. For a more definitive result, it would be ideal for the worms to be on RNAi for longer, given strength of RNAi can increase over generations. Alternatively, a *calm*-1 loss of function mutant could also be attempted. It is a bit unusual that the number of uncoordinated *mir-51(-)* worms did not seem to exhibit any dose dependence. Assays will be re-run with higher sample sizes and blinded scoring. Additionally, experiments will be run with additional concentrations, varying incubation time, and varying recovery time to optimize the experiment. Then, assays will be run on more miRNA mutant strains to see if this differing sensitivity is specific to *mir-51(-)* worms. Once assays are re-run with the potassium channel agonists in a lower DMSO concentration, both *twk-12* and *twk-28* can be knocked down if any sensitivity results from these agents.

In addition to completing the ion assays with potassium agents, generating more transgenic lines through microinjection of both *calm-1* and *twk-12* 3'UTR reporter constructs, and optimizing pharmacological assays, there are a number of remaining directions for this project. At this point, it is unclear if the genes of interest are truly functioning in body wall muscle; they could also have a neurological function. Tissue-specific rescues of miR-51 could help to answer this question. Other remaining questions would be which particular genes of

interest are contributing to the observed phenotype, and by what mechanism of action. These questions will involve a closer look at the individual genes themselves.

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